A Comparison of a Prototype PCR Assay and Hybrid Capture 2 for Detection of Carcinogenic Human Papillomavirus DNA in Women With Equivocal or Mildly Abnormal Papanicolaou Smears

Mark Schiffman, MD, MPH,[1] Cosette M. Wheeler, PhD,[2] Abhijit Dasgupta, PhD,[1] Diane Solomon, MD,[3] and Philip E. Castle, PhD, MPH,[1] for the ALTS Group[*]

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Abstract

We evaluated Hybrid Capture 2 (HC2) and polymerase chain reaction (PCR) results for paired specimens collected at 19,187 visits from 5,026 of 5,060 women participating in the Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS). We examined the test agreement between HC2 and PCR detection for any of 13 carcinogenic human papillomavirus types targeted by HC2 and compared clinical performance of the 2 tests for detecting concurrent and follow-up cervical intraepithelial neoplasia (CIN) 3 or cancer.

The κ value for the 2 assays was 0.65 (95% confidence interval, 0.64-0.66), with 82.7% crude agreement. HC2 was more sensitive (93.6% vs 89.3%; $P < .0005$*) but less specific (41.2% vs 48.5%;* $P < .0005$*) than PCR for detecting 2-year cumulative CIN 3 or cancer (*$n = 503$*). The presence of multiple types as detected by PCR and/or cytologic abnormality increased the likelihood of an HC2+ result.*

Increased sensitivity of HC2 compared with PCR was surprising, given the theoretical advantages of PCR-based methods for analytic sensitivity. Smaller amounts of material used in PCR could have limited its sensitivity, but our results demonstrate the importance of optimization and standardization of PCR-based assays for clinical applications.

Human papillomavirus (HPV) DNA testing is cost-effective[1-3] (S. Kulasingam, PhD, et al, unpublished Atypical Squamous Cells of Undetermined Significance [ASCUS]/Low-Grade Squamous Intraepithelial Lesion [LSIL] Triage Study [ALTS] data) for the triage of equivocal Papanicolaou (Pap) smears, called ASCUS. Annually, more than 2 million US women have Pap test findings of ASCUS, making it the most common nonnormal cytologic interpretation.[4] Most women with ASCUS do not have a significant abnormality requiring treatment; however, about 10% have underlying precancer (cervical intraepithelial neoplasia [CIN] grade 3). In fact, in the United States, ASCUS is the most frequent cytologic interpretation preceding histologic diagnoses of CIN 3 and cancer.[5] Triage of women with ASCUS using HPV testing stratifies women according to risk: HPV– women are very unlikely to have CIN 3 or cancer and can be rescreened in a year, avoiding additional procedures, whereas HPV+ women can be referred for colposcopy for further evaluation and possible tissue biopsy.

Hybrid Capture 2 (HC2) (Digene, Gaithersburg, MD) is the only HPV test approved by the US Food and Drug Administration as an adjunct to cytology for screening and for triage of ASCUS cytologic findings. The HC2 probe set B targets a combined group of 13 carcinogenic HPV types that cause the vast majority of cervical cancer worldwide.[6]

DNA amplification methods such as those based on polymerase chain reaction (PCR) are being introduced and soon will offer an alternative to HC2. These methods hold promise because, experimentally, they can be analytically very sensitive and HPV type-specific. Recent analyses have indicated that the clinical value of HPV testing for cancer risk estimation could be improved by separate identification of

selected carcinogenic HPV types because some carcinogenic types pose higher risks than others and because persistent infection with a carcinogenic type is likely to be the critical risk factor for cancer rather than sequential infections with different types.[7]

Several companies have developed PCR assays for commercialization, some of which are being sold in Europe and, presumably, soon will be sold in the United States. Also, clinical laboratories have developed in-house "home-brew" PCR for HPV testing. However, the validity of these PCR methods has not been confirmed against rigorous end points. Translation (ie, acceptance) of molecular diagnostic assays into clinical practice must be based on evidence of test performance in a realistic setting: conceptual appeal and/or research laboratory results are not sufficient.[8]

ALTS was a 2-year randomized clinical trial to evaluate management strategies, including HPV DNA testing, for women with equivocal cytologic findings (ASCUS) and mildly abnormal cytologic findings (LSIL). In ALTS, HC2 and a prototype PCR assay were performed on almost 20,000 cervical specimens obtained throughout the study, permitting a direct comparison of the 2 technologies for detection of specific types of HPV DNA and histologically confirmed CIN 3 or cancer.

Materials and Methods

Study Design and Population

ALTS was a randomized trial conducted by the National Cancer Institute (National Institutes of Health, Rockville, MD) comparing 3 triage strategies for women with ASCUS or LSIL; details of the design, methods, and primary results of ALTS have been published elsewhere.[9-13] Briefly, women with ASCUS or LSIL cytologic findings were recruited to participate in the study at 4 clinical centers: University of Alabama at Birmingham, Magee-Women's Hospital of the University of Pittsburgh Medical Center Health System (Pittsburgh, PA), the University of Oklahoma (Oklahoma City), and the University of Washington (Seattle). The National Cancer Institute and local institutional review boards approved the study.

Women were randomized to 3 management strategies: immediate colposcopy (referral for colposcopy regardless of enrollment test results); HPV triage (referral for colposcopy if the enrollment HPV result was positive or missing or if the enrollment cytologic findings were high-grade squamous intraepithelial lesion [HSIL]); conservative management (referral for colposcopy if the cytologic diagnosis at enrollment or during follow-up was HSIL). A total of 5,060 women who were eligible and provided written informed consent were enrolled in the study from November 1996 to December 1998, including 3,488 women with ASCUS (mean age, 28.8 years; median age, 26 years; range, 18-81 years) and 1,572 with LSIL (mean age, 24.8 years; median age, 23 years; range, 18-68 years) cytologic findings. Routine follow-up visits were scheduled every 6 months for the 2-year duration of the study; any woman with HSIL cytology was referred for colposcopy. More than 80% of women underwent an exiting examination and a colposcopic evaluation. Women with CIN 2 or worse or persistent HPV-associated lower-grade abnormalities were offered loop electrosurgical excision procedure (LEEP) at exit. Routine follow-up and exit visits concluded in January 2001.

At enrollment, women in each arm received the same enrollment pelvic examination with collection of 2 cervical specimens, the first in PreservCyt for ThinPrep cytologic examination (Cytyc, Boxborough, MA) and the second in specimen transport medium (Digene, Gaithersburg, MD). Each ALTS participant was administered a questionnaire at enrollment to obtain information on demographics, lifestyle, and medical history. We refer readers to other references for details on randomization, examination procedures, patient management, and laboratory and pathology methods.[9,12,13]

HPV DNA Testing

HC2 using the probe set B (henceforth referred to as HC2) is a DNA test for 13 carcinogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).[14,15] Of note, it includes 1 type, HPV-68, that a recent evaluation by the International Agency for Research on Cancer (IARC) did not include as a proven carcinogen.[16] HPV-68 is not well detected by some PCR-based amplification methods,[17] which might have led to inadequate evidence in the IARC report. HC2 does not target 1 type (HPV-66) in its probe set that was classified as carcinogenic by the IARC.[16]

After liquid-based, ThinPrep cytology slides were prepared, 4-mL aliquots of the residual in the PreservCyt vials were used for HPV DNA testing by HC2. Details of HC2 testing are presented elsewhere[14,15] and are only summarized here. Viral DNA released from cervical cells is hybridized in solution to RNA probes. These hybrids are captured onto the surface of a well coated with an anti–RNA-DNA hybrid antibody. A second antihybrid antibody conjugated to alkaline phosphatase binds to the immobilized hybrid, and this "sandwich" is detected by light emission from a chemiluminescent substrate. Signal strengths in relative light units (RLU) were compared with 1 pg/mL of HPV-16 DNA positive controls (RLU/PC). The Food and Drug Administration–approved 1.0 RLU/PC (~1 pg/mL) was used as the threshold for a positive result.[15]

We also used L1 consensus primer PGMY09/11 PCR amplification and reverse-line blot hybridization for type-specific detection[17] on cervical specimens collected into specimen

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transport medium from each patient. Specimens were thawed, and a 150-µL aliquot was digested by adding 7.5 µL of digestion solution (20 mg/mL of Proteinase K, 10% laureth-12, 20 mmol/L of tris(hydroxymethyl)aminomethane [Tris], and 1 mmol/L of EDTA [pH 8.5]) and incubating at 60°C for 1 hour. DNA from the digested material was precipitated by first adding 1.0 mL of absolute ethanol containing 0.5 mol/L of ammonium acetate and incubating the mixture overnight at –20°C. The precipitated DNA was pelleted by centrifugation (30 minutes at 13,000*g*), with the supernatant discarded immediately, and the crude DNA pellet was dried overnight at room temperature. The pellet was suspended in 50 µL of 20 mmol/L of Tris and 1 mmol/L of EDTA (pH 8.5).

We amplified 5 µL of each crude DNA pellet by using the PGMY09/11 L1 consensus primer system and AmpliTaq Gold polymerase (Perkin Elmer, Wellesley, MA). Amplifications were done in a thermal cycler (model 9600; Perkin Elmer) using the following algorithm: 9-minute AmpliTaq Gold activation at 95°C followed by 40 cycles of 1-minute denaturation at 95°C, 1-minute annealing at 55°C, and 1-minute extension at 72°C, and a 5-minute final extension at 72°C.

PCR amplifications and reverse line blot hybridization using HPV genotyping strips (Roche Molecular Systems, Alameda, CA) to detect 27 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51-59, 66, 68, 73 [PAP238A], 82 subtype [W13b], 83 [PAP291], and 84 [PAP155]) and a β-globin internal control were performed by laboratory 1 on the enrollment specimens from the first approximately 2,000 women enrolled into ALTS. Subsequently, PCR amplifications and genotyping for 38 types, including 11 additional noncarcinogenic genotypes (61, 62, 64, 67, 69-72, 81, 82 subtype [IS39], and 89 [CP6108]), were performed by laboratory 2 using genotyping strips produced in 1 lot (by C.M.W.) in collaboration with Roche. We combined the PCR results from the 2 laboratories after an analysis revealed similar performance by each on enrollment specimens **Table 1**. For our main analyses, to simulate a PCR test targeting the same 13 carcinogenic types targeted by HC2, PCR results were considered positive if at least 1 of the 13 carcinogenic types was detected.

Pathology

Clinical management was based on the clinical center pathologists' cytologic and histologic diagnoses. In addition, all referral smears, ThinPrep specimens, and histologic slides were sent to the Pathology QC group (QC pathology) based at Johns Hopkins Hospital, Baltimore, MD, for review diagnoses.

Analysis

HC2 and PCR tests were done on samples obtained from each individual at baseline and at 4 follow-up times (6, 12, 18, and 24 months). This repeated measurement generated "autocorrelation" in the test results for a given woman for the 5 time points, reducing the variance of the results compared with an equal number of strictly independent measurements. The present article is concerned primarily with the comparison of HC2 and PCR, and this comparison was not affected by autocorrelation. Specifically, when we examined the data by follow-up visit time to create data subsets with only 1 set of data per woman, the κ value between the two tests was fairly constant over time (0.67, 0.61, 0.60, 0.59, and 0.64), as was the total agreement percentage (83.8%, 81.2%, 81.0%, 81.7%, and

Table 1
Paired HC2 and PCR Results for Any of 13 HPV Genotypes Targeted by HC2, Among Enrollment Specimens, by PCR Testing Laboratory, Stratified by Trial Arm and Referral Pap Smear Interpretation*

	Laboratory 1				Laboratory 2				
	HC2–		HC2+		HC2–		HC2+		
Trial Arm/Referral Pap Interpretation	PCR–	PCR+	PCR–	PCR+	PCR–	PCR+	PCR–	PCR+	*P*†
Immediate colposcopy									
ASCUS	98 (43.9)	10 (4.5)	24 (10.8)	91 (40.8)	339 (40.6)	45 (5.4)	74 (8.9)	376 (45.1)	.5
LSIL	77 (13.2)	12 (2.1)	84 (14.4)	412 (70.4)	5 (11.0)	2 (4.0)	6 (13.0)	32 (71.0)	.7
HPV triage									
ASCUS	100 (45.7)	5 (2.3)	25 (11.4)	89 (40.6)	350 (40.8)	51 (5.9)	86 (10.0)	371 (43.2)	.1
LSIL	25 (12.2)	5 (2.4)	32 (15.6)	143 (69.8)	2 (33.0)	0 (0.0)	0 (0.0)	4 (67.0)	.4
Conservative management									
ASCUS	92 (41.1)	9 (4.0)	22 (9.8)	101 (45.1)	371 (43.9)	47 (5.6)	82 (9.7)	346 (40.9)	.6
LSIL	92 (15.6)	7 (1.2)	81 (13.7)	411 (69.5)	6 (14.0)	1 (2.0)	5 (12.0)	30 (71.0)	.9

ASCUS, atypical squamous cells of undetermined significance; HC2, Hybrid Capture 2; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; Pap, Papanicolaou; PCR, polymerase chain reaction; +, positive; –, negative.

* Data are given as number (percentage). Numbers of cases were as follows: Laboratory 1: immediate colposcopy, ASCUS, 223; LSIL, 585; HPV triage, ASCUS, 219; LSIL, 205; conservative management, ASCUS, 224; LSIL, 591; Laboratory 2: immediate colposcopy, ASCUS, 834; LSIL, 45; HPV triage, ASCUS, 858; LSIL, 6; conservative management, ASCUS, 846; LSIL, 42. For proprietary information, see the text.

† Pearson χ^2 was used to test for differences between laboratories in the distribution of paired test results for each stratum defined by referral Papanicolaou and study arm.

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84.4%). Our subsequent analyses reflect the association between all paired HC2 and PCR tests, and, as such, pooling the association measures over the time points does not bias our conclusions and, in fact, adds to the precision of our analysis.

Paired test results corresponding to 19,187 visits for 5,026 women (99.3% of all women) were considered in these analyses. Thirty-four women were excluded because they did not have paired HPV testing results at any time during the study. Of the 5,026 women, 1,423 (28.3%) had 4 visits and 1,971 (39.2%) had 5 visits (9 women had more than 5 visits owing to repeated visits) that contributed to this comparison of assays; 4,682, 3,745, 3,550, 3,513, and 3,639 women contributed data to time bins 0, 6, 12, 18, and 24 months, respectively.

The κ values with 95% confidence intervals (CIs) and total and positive agreement percentages were calculated as measures of test agreement between HC2 and PCR detection of at least 1 of the 13 carcinogenic types targeted by HC2. The McNemar χ^2 was calculated to test for statistical differences ($P < .05$) in the number of test-positives for paired results.

For each visit, we calculated the clinical sensitivity and specificity of the HC2 and PCR test results for the detection of 2 complementary end points: QC pathology panel diagnoses of CIN 3 or cancer (NOTE: Seven cases of cancer were diagnosed in ALTS.), the trial surrogate end point for cancer risk, and clinical center pathology diagnoses of CIN 2 or worse, the clinical threshold for treatment. Because HPV DNA detection is decreased greatly in the months following treatment, the accuracy of testing can be calculated only for disease that is not yet treated. Therefore, we calculated for each visit period the performance of the HPV tests in detecting concurrent and subsequent diagnoses of CIN 3 or cancer by the QC pathology panel or CIN 2 or worse by clinical center pathologists, excluding women who had undergone treatment by LEEP. (NOTE: Other analyses included these patients because the relationships between the 2 assays, not the relationship between disease end points, were being evaluated.) There were 503, 213, 170, 145, and 126 concurrent and subsequent QC pathology diagnoses of CIN 3 or cancer relevant to HPV tests performed on specimens obtained at enrollment and at 6, 12, 18, and 24 months, respectively. There were 872, 357, 278, 249, and 232 concurrent and subsequent clinical center pathology diagnoses of CIN 2 or worse relevant to HPV tests performed on specimens obtained at enrollment and at 6, 12, 18, and 24 months, respectively. The McNemar χ^2 was calculated to test for statistical differences in sensitivity and specificity between tests.

We also calculated the clinical performance (sensitivity, specificity, and negative and positive predictive values) for the 2-year cumulative cases of CIN 3 or cancer diagnosed by the QC pathology group for enrollment PCR when 4 additional, possibly carcinogenic HPV types (26, 66, 73, and 82 subtype [W13b])[18] were included or all types detected by PCR were included in the definition of a positive test result. (NOTE: The 82 subtype [IS39] was rare and did not appreciably alter estimates of clinical performance and, therefore, was not included in this analysis.) These calculations were repeated for the clinical center diagnoses of CIN 2 or worse. Finally, to rule out any effect of missing specimens, we recalculated the performance of HC2 and PCR detection of 13, 17, or all types to include all tests performed, treating missing values as missing or, separately, as positive.

To examine type specificity of HC2, we compared the percentage of HC2 positivity by each HPV type detected by PCR. Multiple infections were common, necessitating several analytic approaches to clarify possible cross-reactivity. First we calculated the percentage of HC2 positivity for each type regardless of what other types might be present. We then divided multiple infections into 2 groups, 1 group that included 1 or more carcinogenic types in addition to the type being considered and the other that included 1 or more untargeted (noncarcinogenic) types in addition to the type being considered. Finally, we evaluated HC2 positivity vs single-type infections. A separate analysis reexamined the percentage of HC2 positivity by each HPV type detected by PCR for single-type infections, stratified on cytologic interpretations of ThinPrep cytology. For these cytologic interpretations, we relied primarily on the QC pathology group's ratings, using clinical center pathology interpretations (n = 5,432 [28.3%]) only when QC pathology interpretations were not performed or missing. (NOTE: Of the 7,804 cytologic specimens called nonnormal, 1,697 [21.7%] were based on clinical center pathology's cytologic interpretations.)

Results

Carcinogenic HPV Detection

Of the 19,187 specimens from 5,026 women, 11,166 specimens (58.2%) tested PCR+ for any type, and 53.2% of those positive for any HPV type were positive for multiple types. Most specimens (n = 7,833 [70.2%]) that tested positive for any HPV type by PCR were positive for at least 1 of the 13 HPV types targeted by HC2, and 34.6% of those positive for any of the 13 types were positive for more than 1 of the 13 types. By comparison, 8,783 specimens tested positive by HC2. The greater positivity of HC2 compared with PCR for 13 types (8,783 vs 7,833) was statistically significant in paired testing ($P < .0001$), with a κ of 0.65 (95% CI, 0.64-0.66), a total agreement of 82.7%, and positive agreement of 66.7% **Table 2**. Among the HC2+ samples, testing positive by PCR was associated strongly with greater HC2 signal strength ($P_{Trend} < .0001$), a surrogate for viral load.[19] Among PCR+

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samples, testing positive by HC2 was associated strongly with the number of PCR-detected types ($P_{Trend} < .0001$), with the number of PCR-detected carcinogenic types ($P_{Trend} < .0001$), and with the number of PCR-detected noncarcinogenic types ($P_{Trend} < .0001$). There was an increasingly high percentage of specimens negative for both HC2 and PCR (for the 13 types) at later study visits ($P_{Trend} < .0001$, data not shown), a trend that is consistent with infections clearing over time and/or successful treatment of lesions that were CIN 2 or worse.

Detection of CIN 2 or Worse and CIN 3 or Cancer

We next compared PCR and HC2 results in relation to 2 disease end points, cumulative clinical center pathology diagnoses of CIN2 or worse, the clinical threshold for treatment, and cumulative QC pathology diagnoses of CIN 3 or cancer, a more rigorous definition of cervical cancer risk **Table 3**. We found that at any time point in the study (Note: In this analysis, women who were treated by LEEP were excluded from evaluations at later time points.), HC2 compared with PCR was more sensitive but less specific for detection of disease using either definition. For example, HC2 was more sensitive (93.6% vs 89.3%; $P = .0004$) and less specific (41.2% vs 48.5%; $P < .0001$) than PCR as performed on enrollment specimens for detection of the 2-year cumulative CIN 3 or cancer cases diagnosed by QC pathologists. Similarly, HC2 was more sensitive (93.7% vs 87.2%; $P < .0001$) and less specific (44.6% vs 51.7%; $P < .0001$) than the PCR performed on enrollment specimens for the detection of the 2-year cumulative CIN 2 or worse cases diagnosed by clinical center pathologists. We excluded visits at which the clinicians knew HPV positivity or negativity by HC2 to assess any bias

Table 2
Comparison of HC2 Test Results and PCR Detection of Any of 13 HPV Genotypes Targeted by HC2*

	PCR Results		
HC2 Results	**Negative**	**Positive**	**Total**
Negative	9,220 (48.1)	1,184 (6.2)	10,404
Positive	2,134 (11.1)	6,649 (34.7)	8,783
Total	11,354	7,833	19,187

HC2, Hybrid Capture 2; HPV, human papillomavirus; PCR, polymerase chain reaction.
* Data are given as number (percentage based on the overall total). HPV types were 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. $\kappa = 0.65$ (95% confidence interval, 0.64-0.66); total agreement, 82.7%; positive agreement, 66.7%; $P < .0001$, McNemar χ^2. For proprietary information, see the text.

Table 3
Comparison of Clinical Performance for the Detection of CIN 3 or Cancer and CIN 2 or Worse by HC2 and PCR Detection of Any of 13 Carcinogenic HPV Genotypes Targeted by HC2*

		HC2–		HC2+		CIN 3 or Cancer‡			CIN 2 or Worse§			
Visit	**N†**	**PCR–**	**PCR+**	**PCR–**	**PCR+**	**No. of Cases**	**Sensitivity (%)**	**Specificity (%)**	**No. of Cases**	**Sensitivity (%)**	**Specificity (%)**	**Referral (%)**
1, enrollment	4,682	1,559	194	521	2,408	503			872			
HC2							93.6	41.2		93.7	44.6	62.6
PCR							89.3	48.5		87.2	51.7	55.6
P							**.0004**	**<.0001**		**<.0001**	**<.0001**	
2, 6 mo	3,360	1,548	223	392	1,197	213			357			
HC2							93.4	55.8		89.9	57.8	47.3
PCR							85.0	60.6		80.1	62.2	42.3
P							**.0007**	**<.0001**		**<.0001**	**<.0001**	
3, 12 mo	3,083	1,533	204	363	983	170			278			
HC2							92.9	59.2		91.7	61.1	43.7
PCR							86.5	64.3		84.5	66.1	38.5
P							**.02**	**<.0001**		**.0009**	**<.0001**	
4, 18 mo	3,009	1,602	186	357	864	145			249			
HC2							91.7	62.0		91.6	64.0	40.6
PCR							89.0	67.8		85.5	69.7	34.9
P							.3	**<.0001**		**.01**	**<.0001**	
5, 24 mo	3,063	1,789	170	314	790	126			232			
HC2							84.9	66.1		87.5	68.2	36.0
PCR							81.7	70.8		80.6	72.7	31.3
P							.4	**<.0001**		**.009**	**<.0001**	

CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; HPV, human papillomavirus; PCR, polymerase chain reaction; +, positive; –, negative.
* The case definition included concurrently and subsequently diagnosed cases to the time of the visit and the collection of specimens for HPV DNA testing. Women who underwent loop electrosurgical excision procedure or were lost to follow-up were not included in subsequent calculations. The McNemar χ^2 test was used as the statistical test to test for differences in clinical sensitivity and specificity. *P* values < .05 were considered statistically significant and are indicated in bold type. For proprietary information, see the text.
† Number of paired tests.
‡ Number of cases diagnosed by ALTS (Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study) Pathology Quality Control Group from the indicated time point to the end of the study.
§ Number of cases diagnosed by clinical center pathologists from the indicated time point to the end of the study.

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but found none. Specifically, exclusion of enrollment cases diagnosed in the HPV arm, in which colposcopic referral was based on a positive HC2 test result, and cases diagnosed at exit when HPV data were partially unmasked for all arms did not appreciably change our conclusions (data not shown).

The addition of 4 more possibly carcinogenic types[18] detected to the definition of a PCR+ test resulted in a test that was still significantly less sensitive and more specific for the detection of 2-year cumulative CIN 3 or cancer compared with HC2 **Table 4**. The inclusion of all types detected into the definition of a PCR+ test resulted in a similarly sensitive (92.8% for PCR vs. 93.6% for HC2) but significantly less specific test for the detection of 2-year cumulative CIN 3 or cancer compared with HC2. Similar results were observed for analyses that used 2-year cumulative CIN 2 or worse cases diagnosed by clinical center pathologists as the disease end point (data not shown). There was little difference in sensitivity and specificity of either assay when all tests performed were considered compared with the restricted set of women with both tests. Treating missing test results as positive did not appreciably increase sensitivity but decreased specificity nonsignificantly by 1% to 2% (Table 4).

HC2 Reactivity With PCR-Detected Types

We next considered the impact of PCR-detected types on testing positive by HC2. For the specimens in which only 1 type was detected by PCR, 12 of 13 types targeted by HC2 were among the 14 types with the highest percentage of HC2+ results (88.3%-64.5%) **Table 5**; single-type infections with HPV-68 were only 19th on the list at 58% HC2+. Although not targeted by specific HC2 probes, 80.0% of single-type infections by the carcinogenic type HPV-66 tested positive by HC2. Eighty percent of the single-type HPV-82 subtype [IS39] infections tested positive, but this type was found rarely. At least 50% of single-type infections by untargeted types (in decreasing order of percentage of HC2+) HPV-82 subtype [W13b], 70, 26, 67, and 53 tested HC2+.

For each single type that we evaluated (whether one of the 13 targeted types or not), HC2 positivity was increased significantly if at least 1 additional type of those targeted by HC2 also was present, creating a multitype infection (Table 5, second compared with fourth percentage columns). The only exception was HPV-57, which was virtually never found alone. The presence of at least 1 additional untargeted type in a multitype infection (Table 5, third percentage column) also increased the likelihood of a positive HC2 test result, particularly for types that were less apt to test positive for HC2 when found alone. This increase was significant for targeted HPV types 59, 45, and 68 and for untargeted types 82 subtype [W13b], 53, 42, 73, 55, 84, 61, 62, 54, 83, 72, 89 [CP6108], 40, and 81.

For single-type infections, there was a greater tendency of HC2 testing positive with increasing severity of the interpretation of the ThinPrep cytologic slide **Table 6**. This trend was significant for all types except for targeted type HPV-33 and for untargeted types 82 subtype [IS39], 82 subtype [W13b], 26, 69, 71, 64, 11, 81, and 57.

Discussion

In ALTS, dual testing by a prototype commercial PCR assay and HC2 was performed on specimens obtained at nearly 20,000 visits from approximately 5,000 patients during 2 years, thereby permitting a robust comparison of the 2 tests.

Table 4
Comparison of the Clinical Performances for the Detection of 2-Year Cumulative Cases of CIN 3 or Cancer by PCR and HC2*

	PCR									HC2		
	13 HPV Types			17 HPV Types			All HPV Types					
	All + Miss	All	Paired	All + Miss	All	Paired	All + Miss	All	Paired	All + Miss	All	Paired
ASCUS Referral Pap												
Sensitivity (%)	**87.3**	**86.7**	**87.4**	**88.6**	**88.1**	**88.5**	92.2	91.8	91.7	92.8	92.4	92.5
Specificity (%)	53.7	55.7	55.6	50.7	52.6	52.6	**39.9**	**41.4**	**41.5**	48.3	50.6	51.1
PPV (%)	15.4	15.7	15.8	14.7	15.1	15.1	12.9	13.0	13.0	14.7	15.2	15.2
NPV (%)	97.8	97.8	97.9	97.9	97.9	98.0	98.2	98.2	98.1	98.6	98.6	98.6
All participants												
Sensitivity (%)	**89.1**	**88.8**	**89.3**†	**90.4**	**90.1**	**90.5**	93.0	92.8	92.8	93.9	93.6	93.6†
Specificity (%)	47.3	48.7	48.5†	43.6	44.9	44.8	**33.5**	**34.5**	**34.6**	39.0	41.0	41.2†
PPV (%)	16.9	17.2	17.3	16.1	16.4	16.5	14.4	14.5	14.6	15.6	16.1	16.1
NPV (%)	97.3	97.3	97.4	97.4	97.4	97.5	97.6	97.6	97.6	98.2	98.2	98.2

CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; HPV, human papillomavirus; PCR, polymerase chain reaction.
* Several definitions of test positive were considered for PCR: 13 carcinogenic HPV types targeted by HC2 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), 17 types (13 HC2-targeted carcinogenic types plus 26, 66, 73, 82 subtype [W13b]), and all types detected. Clinical performance, as measured by sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV), is shown for the subset that had both test results (Paired), for all test results (All), and for all test results and treating missing results as positive (All + Miss). Bold and underlined type indicates statistically significant lesser and greater values, respectively, for PCR compared with HC2. For proprietary information, see the text.
† Results given in Table 3.

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We found moderate to good agreement on test positivity when we compared HC2 with PCR detection of the 13 types targeted by HC2. However, HC2 was more likely to test positive compared with PCR detection of the 13 types. HC2 demonstrated increased clinical sensitivity (more test-positives among cases) but lower specificity (more test-positives among noncase samples) than PCR for detection of cervical precancer. If clinical sensitivity and specificity are considered equally important aspects of overall accuracy, the 2 tests were similarly accurate.

The lower clinical sensitivity of PCR for CIN 3 or CIN 2 compared with HC2 was surprising, given the theoretical advantage of DNA amplification methods to achieve greater analytic sensitivity. The greater sensitivity of HC2 vs PCR might relate to unintended but fortunate cross-reaction of HC2 with types that occasionally might cause cancer, like HPV-66,[18] and/or the detection of infections by more strongly carcinogenic types (ie, the 13 carcinogenic types) that were missed by PCR. We observed evidence for both explanations.

The cross-reactivity of HC2 with certain untargeted types like HPV-66, especially in the context of cytologic abnormalities, has been reported[20,21] and confirmed in this study. However, the addition of HPV-66 and other potentially carcinogenic HPV types (26, 73, and 82 subtype [W13b])[18] into the definition of a positive test for carcinogenic HPV did not significantly improve the sensitivity of detection of CIN 3 or cancer (Table 4) while decreasing specificity, as previously reported.[22] Even the inclusion of all types detected by PCR, including noncarcinogenic types as surrogates for coinfected but possibly missed carcinogenic types, in the definition of a

Table 5
HC2+ Test Results Among PCR+ Results for an HPV Type for All Infections, Infections Including at Least One Additional HC2-Targeted Type, Infections Including at Least One Additional Untargeted Type, and Single-Type Infections*

	Any		+ ≥ 1 Targeted Type (Multitype)		+ ≥ 1 Untargeted Type (Multitype)		Single Type	
HPV Type	N†	HC2+ (%)	N	HC2+ (%)	N	HC2+ (%)	N	HC2+ (%)
35‡	697	90.5	401	95.0	134	79.9	162	88.3
56‡	672	93.3	378	96.0	137	92.0	157	87.9
31‡	946	92.1	516	95.7	181	89.5	249	86.4
51‡	935	90.4	548	94.2	191	84.3	196	85.7
33‡	432	89.4	259	92.7	81	84.0	92	84.8
16‡	2,079	87.9	1,020	93.4	451	83.2	608	82.1
66	638	91.1	419	95.5	104	85.6	115	80.0
82 [IS39]	47	89.4	28	96.4	9	77.8	10	80.0
58‡	810	87.4	462	92.2	149	82.6	199	79.9
39‡	850	87.5	514	93.2	161	78.3	175	79.4
18‡	877	86.6	496	93.6	136	77.9	245	77.1
52‡	1,465	84.4	788	93.0	294	77.2	383	72.1
59‡	767	87.2	454	95.6	160	83.1	153	66.7
45‡	662	84.7	409	94.4	112	75.0	141	64.5
82 [W13b]	257	88.7	182	94.5	36	88.9	39	61.5
70	505	76.6	249	89.6	133	67.7	123	60.2
26	108	78.7	59	89.8	29	69.0	20	60.0
67	302	83.1	182	94.0	61	73.8	59	59.3
68‡	477	83.2	302	91.7	94	77.7	81	58.0
53	1,036	78.3	622	90.2	209	66.5	205	54.2
6	454	74.7	272	91.9	83	50.6	99	47.5
69	50	72.0	34	85.3	7	57.1	9	33.3
42	681	69.0	394	90.6	125	48.0	162	32.7
73	384	69.8	247	86.6	71	47.9	66	30.3
71	106	67.0	68	89.7	8	37.5	30	23.3
55	455	66.6	282	88.7	83	39.8	90	22.2
64	51	70.6	35	82.9	11	54.6	5	20.0
84	656	60.1	359	84.1	150	42.0	147	19.7
61	858	59.1	462	85.1	168	45.8	228	16.2
62	1,112	58.5	606	85.0	253	38.7	253	14.6
54	933	61.2	547	86.1	167	40.7	219	14.6
83	722	62.5	412	87.1	163	43.6	147	14.3
72	214	50.5	108	73.2	56	39.3	50	14.0
89	767	60.4	444	85.1	159	40.3	164	12.8
40	281	69.4	192	89.6	45	42.2	44	9.1
11	108	73.2	84	89.3	12	25.0	12	8.3
81	397	58.7	213	84.0	100	47.0	84	8.3
57	11	81.8	6	100.0	4	75.0	1	0.0

HC2, Hybrid Capture 2; HPV, human papillomavirus; PCR, polymerase chain reaction; +, positive.
* Types were ordered according to the percentage HC2+ for single-type infections (last column). For proprietary information, see the text.
† Numbers in this column represent the sum of the 3 other number columns.
‡ Types targeted by HC2.

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positive test approached but did not achieve the sensitivity of HC2 with a concomitant large decrease in specificity. Thus, we suggest that the greater clinical sensitivity of HC2 compared with PCR was due in part to truly greater analytic sensitivity for the 13 carcinogenic types targeted by HC2. We directly confirmed this conclusion by examining the longitudinal patterns of HPV positivity for the 2 assays among women diagnosed with CIN 3 or cancer after enrollment. We found cases of initial PCR negativity and HC2 positivity, in which subsequently obtained specimens were PCR+ for the 13 carcinogenic types at multiple, sequential visits leading to diagnosis of CIN 3 (data not shown).

It is noteworthy that HC2 detection of targeted types was weakest for HPV-68, the same type that is detected poorly by certain PCR primer systems.[17] HPV-68 has long been identified as one of the carcinogenic types.[6] Recent studies have raised questions as to the carcinogenic potential of HPV-68, which was left off the list of carcinogenic types by a recent consensus meeting document.[16] We note that in ALTS, the absolute risk for CIN 3 or cancer for single-type infections by HPV-68 was 7.7% (95% CI, 0.9%-25.1%, binomial exact), which was much less than HPV-16 (39.1%; 95% CI, 32.9%-45.7%)[7] but greater than other targeted types like HPV-56 (1.9%; 95% CI, 0.0%-9.9%) and HPV-59 (0.0%; 95% CI, 0.0%-8.2%) and the untargeted but potentially carcinogenic HPV-66 (3.8%; 95% CI, 0.5%-13.2%). The importance of HPV-68 detection remains to be clarified, given its relatively poor detection by several assay systems, and the value of its inclusion in future clinical assays requires further evaluation once its detection has been optimized.

Table 6
HC2+ Test Results Among Single-Type Infections Detected by PCR Stratified on Cytologic Interpretation by the Quality Control Pathology Group*

	Negative		ASCUS		LSIL		HSIL or Worse	
HPV Type	**N**	**HC2+ (%)**	**N**	**HC2+ (%)**	**N**	**HC2+ (%)**	**N**	**HC2+ (%)**
35†	62	77.4	38	86.8	43	100.0	19	100.0
56†	54	68.5	37	94.6	62	100.0	4	100.0
31†	109	74.3	68	92.7	43	100.0	26	100.0
51†	68	69.1	40	87.5	73	97.3	14	100.0
33†	42	76.2	21	90.5	17	94.1	11	90.9
16†	232	65.5	188	87.2	93	97.9	93	97.9
66	53	66.0	23	82.6	37	100.0	2	50.0
82 [IS39]	2	0.0	3	100.0	2	100.0	3	100.0
58†	78	56.4	59	91.5	44	97.7	18	100.0
39†	91	65.9	31	87.1	49	100.0	4	75.0
18†	99	64.7	68	80.9	58	89.7	19	89.5
52†	207	59.4	104	81.7	51	96.1	19	94.7
59†	80	50.0	37	81.1	31	93.6	3	66.7
45†	78	52.6	30	56.7	26	100.0	6	100.0
82 [W13b]	19	47.4	10	70.0	6	83.3	2	100.0
70	84	53.6	23	65.2	13	84.6	3	100.0
26	8	37.5	2	100.0	6	66.7	4	75.0
67	26	38.5	23	65.2	6	100.0	4	100.0
68†	53	49.1	18	77.8	8	75.0	1	100.0
53	118	36.4	44	65.9	43	90.7	0	—
6	46	21.7	11	45.5	40	80.0	0	—
69	7	28.6	0	—	1	100.0	1	0.0
42	89	16.9	40	25.0	33	84.9	0	—
73	36	22.2	14	21.4	11	54.6	5	60.0
71	23	17.4	4	50.0	2	50.0	1	0.0
55	66	15.2	22	36.4	2	100.0	0	—
64	3	33.3	0	—	1	0.0	1	0.0
84	108	16.7	28	17.9	11	54.6	0	—
61	168	14.3	47	17.0	9	55.6	0	—
62	200	11.5	42	19.1	9	66.7	1	0.0
54	164	10.4	43	18.6	10	60.0	1	100.0
83	126	13.5	16	6.3	4	50.0	1	100.0
72	38	10.5	7	28.6	4	25.0	1	0.0
89	133	9.8	26	19.2	3	66.7	2	50.0
40	28	0.0	10	20.0	5	40.0	0	—
11	5	0.0	4	25.0	3	0.0	0	—
81	59	8.5	19	5.3	5	0.0	1	100.0
57	0	—	1	0.0	0	—	0	—

ASCUS, atypical squamous cells of undetermined significance; HC2, Hybrid Capture 2; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; PCR, polymerase chain reaction; +, positive.
*Types were ordered according to the percentage HC2+ for single-type infections (Table 5, last column). For proprietary information, see the text.
†Types targeted by HC2.

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We also observed an overall trend of lower sensitivity for either HPV assay for detection of cumulative cases of CIN 3 or cancer or CIN 2 or worse for testing performed on specimens from later visits, with a large decrease for sensitivity for CIN 3 cases diagnosed at the exit visit (eg, HC2 detection of CIN 3 or cancer: 93.6%, 93.4%, 92.9%, 91.7%, and 84.9% for visits 1 through 5, respectively). The reason for the decrease is unclear, but we hypothesize that with intensive screening, only the smallest CIN 3 lesions remained untreated and, therefore, were more likely to be missed by any screening test.[23] Alternatively, some lesions were misclassified possibly as CIN 3. Indeed, 4 exit cases tested negative for both assays at every visit for which we had test results, and a fifth case was negative by PCR at all 5 visits and negative by HC2 at 4 of 5 visits, with a single positive HC2 test at enrollment. We also note that concurrent ThinPrep cytologic findings with CIN 3 cases diagnosed at the exit visit in the study were more likely to be called negative (22.8%) than for CIN 3 cases diagnosed at any other visit (8.4%; $P < .0005$; Pearson χ^2), which is consistent with the smaller lesions detected at exit from the study compared to earlier in the study.

We also found that the exit cases of CIN 3 or cancer diagnosed by the QC pathology group were less likely to have a concurrent clinical pathologist's diagnosis of CIN 2 or worse than cases diagnosed during enrollment and follow-up ($P = .005$; Pearson χ^2). Thus, exit cases were more likely to include a few questionable diagnoses, possibly overcalled by the QC pathology group, which we find plausible given the known uncertainties of even expert histologic diagnoses.[24]

There are several limitations of this comparison that deserve careful consideration. First, the 2 tests were run on aliquots from different specimen collections. HC2 was performed on PreservCyt specimens and PCR on specimens in specimen transport medium, which always were collected second. The impact of this order on HPV testing has not been evaluated. Second, the fraction of specimen used in HC2, 10%, was much greater than the 1.5% used for PCR amplification. Thus, there was likely more viral DNA in aliquots used for HC2 than aliquots used for PCR. However, DNA isolated from cervicovaginal specimens may contain inhibitors of PCR amplification.[25] Thus, we cannot simply assume that increasing the fraction of specimen in PCR amplification reactions would translate to greater analytic or clinical sensitivity. It will be critical to establish optimal reaction parameters, including specimen input and purity, before the commercialization of any new HPV assay.

Two additional factors bear mentioning when comparing the results of the 2 tests. HC2 was performed in 4 clinical laboratories, whereas PCR was performed primarily by 1 expert laboratory. Thus, the HC2 results represent average performance, whereas PCR results do not. Moreover, a single batch of line blots for genotyping PCR amplicons was used by laboratory 2 throughout the study (~90% of all PCR tests). Thus, it is unclear how broadly applicable the PCR results observed in this study will be. Future versions of this PCR assay will be performed by less expert laboratories, but reagents might be more standardized if quality control is excellent. The net impact of these 2 opposing effects on the average performance and overall reliability of the next generation PCR assay cannot be predicted.

We acknowledge that our population, women enrolled into the study with equivocal (ASCUS) or mildly abnormal (LSIL) cytologic findings, is not representative of the entire population of women with HPV infections. Not all women with HPV infections have concurrent cytologic abnormalities. Specimens from women with nonnormal cytologic findings tend to have greater HC2 signal strength,[15] a surrogate for HPV viral load,[19] than specimens from women with normal cytologic findings. As a consequence, there was high test positivity for each assay and, perhaps, a greater concordance between tests than would be expected for a true population sample or for a sample of women with normal cytologic findings.[26] That is, in populations with lower mean HPV viral loads, we anticipate lower concordance between tests and lower (clinical) sensitivity but greater specificity for any HPV DNA assay.

One disadvantage of HC2 testing compared with PCR testing is that HC2 detects any of 13 oncogenic HPV types and, therefore, cannot distinguish the HPV genotypes present. Genotyping can permit detection of persistent HPV infection, a risk factor for progression,[27] and the identification of women with evidence of HPV viral persistence rather than a single positive HPV test result might improve the clinical performance of HPV DNA testing for the detection of cervical neoplasia.[28] It is noteworthy that repeated HC2 positivity often might represent HPV viral persistence. In ALTS, more than 50% of women who had tested positive with HC2 again at 6 months also had repeated PCR+ results for at least 1 of the same 13 oncogenic types (data not shown), although this likely represents an underestimate of the HPV persistence among the double HC2+ samples because of the analytic insensitivity of the PCR assay used in this study to classify infections as persistent.

The detection of carcinogenic HPV DNA by HC2 and by prototype PCR assay performed similarly for the detection of CIN 3 (and CIN 2), with the PCR assay trading off sensitivity for specificity compared with HC2. The goal of the next generation of assays might be to achieve or exceed the clinical sensitivity of HC2 while improving clinical specificity, ie, decreasing the detection of infections not related to risk of cervical precancer (histologically confirmed CIN 3) or cancer. The best approach to meeting the goal is the most exciting current question in diagnostics in relation to cervical cancer screening and prevention.

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From the Divisions of [1]Cancer Epidemiology and Genetics and [3]Cancer Prevention, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD; and [2]Departments of Molecular Genetics and Microbiology and Obstetrics and Gynecology, University of New Mexico Health Sciences Center, School of Medicine, Albuquerque.

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Address reprint requests to Dr Castle: Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Blvd, Room 7074, EPS MSC 7234, Bethesda, MD 20892-7234.

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[*] The ALTS Group: National Cancer Institute, Bethesda, MD: D. Solomon, project officer; M. Schiffman, co-project officer; P.E. Castle; S. Wacholder, statistician; Clinical Centers: University of Alabama at Birmingham, E.E. Partridge, principal investigator; L. Kilgore, co-principal investigator; S. Hester, study manager; University of Oklahoma, Oklahoma City, J.L. Walker, principal investigator; G.A. Johnson, co-principal investigator; A. Yadack, study manager; Magee-Women's Hospital of the University of Pittsburgh Medical Center Health System, Pittsburgh, PA, R.S. Guido, principal investigator; K. McIntyre-Seltman, co-principal investigator; R.P. Edwards, investigator; J. Gruss, study manager; University of Washington, Seattle, N.B. Kiviat, co-principal investigator; L. Koutsky, co-principal investigator; C. Mao, investigator; Colposcopy Quality Control Group: D. Ferris, principal investigator, Medical College of Georgia, Augusta; J.T. Cox, co-investigator, University of California at Santa Barbara; L. Burke, co-investigator, Beth Israel Deaconess Medical Center Hospital, Boston, MA; HPV Quality Control Group: C.M. Wheeler, principal investigator, University of New Mexico Health Sciences Center, Albuquerque; C. Peyton-Goodall, laboratory manager, University of New Mexico Health Sciences Center; M.M. Manos, co-investigator, Kaiser Permanente, Oakland, CA; Pathology Quality Control Group: R.J. Kurman, principal investigator, Johns Hopkins Hospital, Baltimore, MD; D.L. Rosenthal, co-investigator, Johns Hopkins Hospital; M.E. Sherman, co-investigator, National Cancer Institute, Rockville, MD; M.H. Stoler, co-investigator, University of Virginia Health Science Center, Charlottesville; Westat, Coordinating Unit, Rockville, MD: J. Rosenthal, project director; M. Dunn, data management team leader; J. Quarantillo, senior systems analyst; D. Robinson, clinical center coordinator; Quality of Life Group: Diane Harper, ALTS Quality of Life Group, Dartmouth Medical School; Information Management Services, Silver Spring, MD: B. Kramer, senior programmer/analyst.

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